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PB-284 758

Infection of the European Chafer, 'Amphimallon majalis' by 'Bacillus popilliae': Ultrastructure

U.S. Health Effects Research Lab, Research Triangle Park, N C

3 May 77

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/J-78-020	2. JOURNAL ARTICLE	3. PB 284 758

4. TITLE AND SUBTITLE
Infection of the European Chafer, Amphimallon majalis, by Bacillus popilliae: Ultrastructure

5. REPORT DATE 1977

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Environmental Toxicology Division
Health Effects Research Laboratory
Research Triangle Park, N.C. 27711

10. PROGRAM ELEMENT NO. 1EA615

11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS
Health Effects Research Laboratory RTP, NC
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, N.C. 27711

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE EPA 600/11

15. SUPPLEMENTARY NOTES
Published in: J. Invertebr. Pathol. 31:91-102, 1978

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
insects invertebrates bacillus spores	European chafer	06 F, M, T

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO. OF PAGES 14
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE A02 A01

EPA Form 2220-1 (9-73)

EPA-600/J-78-020
JOURNAL ARTICLE

JOURNAL OF INVERTEBRATE PATHOLOGY 31, 91–102 (1978)

Infection of the European Chafer, *Amphimallon majalis*, by *Bacillus popilliae*: Ultrastructure[1]

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Received May 3, 1977

Electron microscopical studies indicate that midgut alterations observed in larval *Amphimallon majalis* during *Bacillus popilliae* invasion were due to infection of epithelium, wound repair, and hemocytic encapsulation. Hemocytes bearing angular cytoplasmic granules formed the capsule which adhered to the basal lamina subjacent to the mesenteronic lesion. Exocytosis of hemocyte granules into bacilli-containing endocytic invaginations of plasma membranes and vacuoles was observed. These hemocytes as well as mesenteronic cells exhibited bactericidal capabilities.

KEY WORDS: *Amphimallon majalis*, larvae; *Bacillus popillae*, spores; bactericidal; mesenteronic cells; hemocytes; exocytosis; midgut.

INTRODUCTION

In previous papers (Splittstoesser et al., 1973, 1978) the route and mode of *Bacillus popilliae* invasion of the larvae of the European chafer, *Amphimallon majalis*, were described. The site of spore germination was deduced to be the lumen of the alimentary tract, while that of the infection was the regenerative nidi regions of the mesenteron following invasion of the mesenteronic columnar epithelium. Resultant lesions appeared to consist of focal necrosis, desquamation of columnar epithelial cells, and increased activity of regenerative nidi. In many such areas of disturbed mesenteronic epithelium, unusual numbers of noncolumnar cells believed to be derived from regenerative nidi were frequently observed. Concurrently an encapsulating response by certain hemocytes culminated in formation of a capsule subjacent to the epithelial lesion. Additional findings of electron microscopical investigations of these events are reported herein.

MATERIALS AND METHODS

Specimens and procedures were identical to those used in the previous study (Splittstoesser et al., 1978). Specimens were fixed 40 hr after initial exposure to *B. popilliae* spores. Fixation of whole midguts were performed sequentially or simultaneously (Hinde, 1971) with glutaraldehyde and osmium. Embedding was in Spurr's low-viscosity plastic. Thin sections for electron microscopy and thick adjacent sections for purposes of orientation with the light microscope were obtained with a Sorvall MT-2 ultramicrotome. Thin sections were double stained with uranyl acetate and lead citrate. Thick sections were stained with a mixture of auramine O, toluidine blue, and safranin 0 as previously described.

RESULTS

Noncolumnar Cells in Regions of Disturbed Epithelium

Invasion of mesenteronic columnar epithelium by *B. popilliae* bacilli resulted in focal necrosis and cell desquamation which

[1] Journal Paper No. 3070 of the New York State Agricultural Experiment Station, Geneva, N. Y. 14456. This investigation was supported in part by National Science Foundation Grant No. GB-27966X to K. H. Steinkraus and H. Tashiro.

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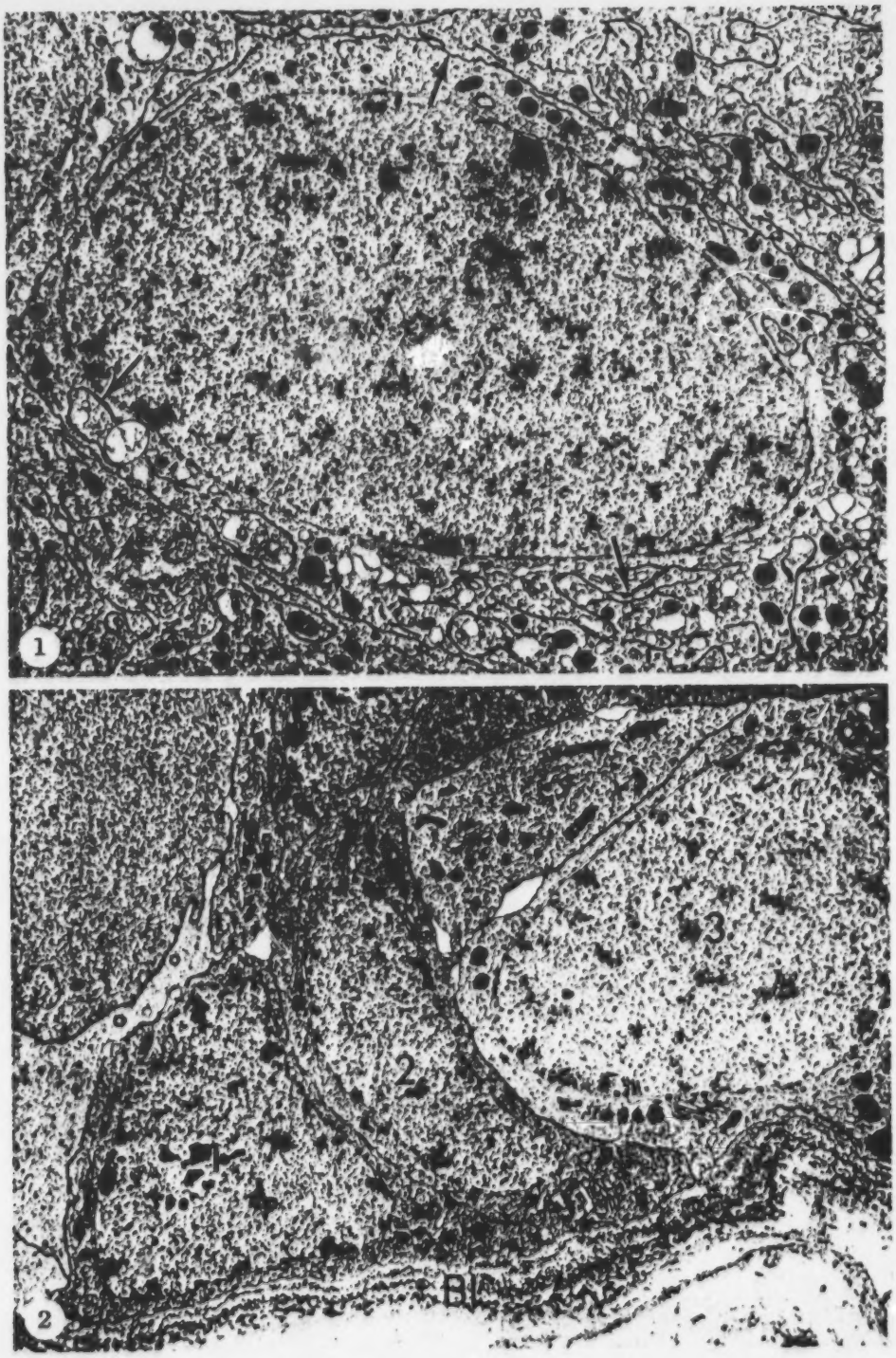


FIGURES 1–2

exceeded the rate of normal turnover (Splittstoesser et al., 1978). These events appeared to increase the mitotic index of regenerative nidi cells within and adjacent to the affected areas. Often within such involved regions at later stages of the infection, increased numbers of noncolumnar epithelial cells appeared. Some were necrotic (previously columnar) epithelial cells while others were of two cytologically definable types. One type (Fig. 1) was ultrastructurally similar to differentiating epithelial cells of the regenerative nidus in control larvae. Long tracts of branching electron-dense membrane and intercellular borders were their most notable characteristics. These cells usually had a moderately high nuclear–cytoplasmic ratio. Nucleoli were often present. Rough endoplasmic reticulum, mitochondria, and Golgi bodies were usually present. Glycogen deposits were occasionally apparent.

The second type of cell (Fig. 2) was similar to the undifferentiated cells of the regenerative nidi. Usually located adjacent to or within proximity of the basal lamina, these cells generally were smaller with a limited cytoplasm. Nucleoli were neither as numerous nor as obvious as in the differentiating cell type. Rough endoplasmic reticulum in these cells was limited and ribosomes and polysomes predominated. Small vesicles and residual bodies were often present.

The two cytologically definable types of mesenteronic cells will be referred to henceforth, for the sake of brevity, as immature cells.

Capsules and Inflammatory Hemocytes

Consequent to formation of an infection focus in the mesenteron by bacilli of *B. popilliae*, an inflammatory reaction by certain hemocytes was observed in earlier studies. Extensive infiltration by these cells was observed on the hemocoelic side of the focal lesion. The hemocytes that initially responded covered the subjacent basal lamina, but as influx continued the cells closely cohered to each other as well. Cells proximal to the mesenteron were tightly interdigitated while those at the periphery appeared to be in the process of incorporating into the capsule and were more loosely attached. Ultimately a compact mass of hemocytes attached to the mesenteron-investing basal lamina resulted. This capsule was often histologically heterogeneous because other mesenteron-investing tissues were included.

Hemocytes accommodated themselves very closely to the contours of basal laminas of the mesenteron and other tissues included in the capsule, completely covering the surfaces and filling even the minute pits and irregularities with no intervening spaces at the interfaces (Fig. 3). The basal lamina in encapsulated areas occasionally differed in ultrastructure from that underlying unencapsulated epithelium, but this appearance could be due to a preparation artifact.

In contrast to interfaces with basal lamina the membranes of interdigitated capsule hemocytes appear not to appress directly on each other. Spaces were evident between plasma membranes at their closest approximations. At these appositions an internal network of microfilaments and frequently microtubules were associated with plasma membranes (Fig. 4).

Desmosome-like structures occurred along tracks of juxtaposed plasma membranes between hemocytes in the closely organized regions of the capsules (Fig. 4). These structures were characterized by a widened intermembrane gap filled with fibrous material of moderate electron den-

FIG. 1. Noncolumnar cell observed within mesenteronic lesion which closely resembles differentiating regenerative nidi cells. Arrows indicate some electron-dense membrane tracts characteristic of these cells. ×8800.

FIG. 2. Noncolumnar cells, labeled 1, 2, and 3, from mesenteronic lesion of treated larva, that are cytologically similar to undifferentiated regenerative nidi cells. These cells are usually observed within proximity of the basal lamina (BL). ×8800.

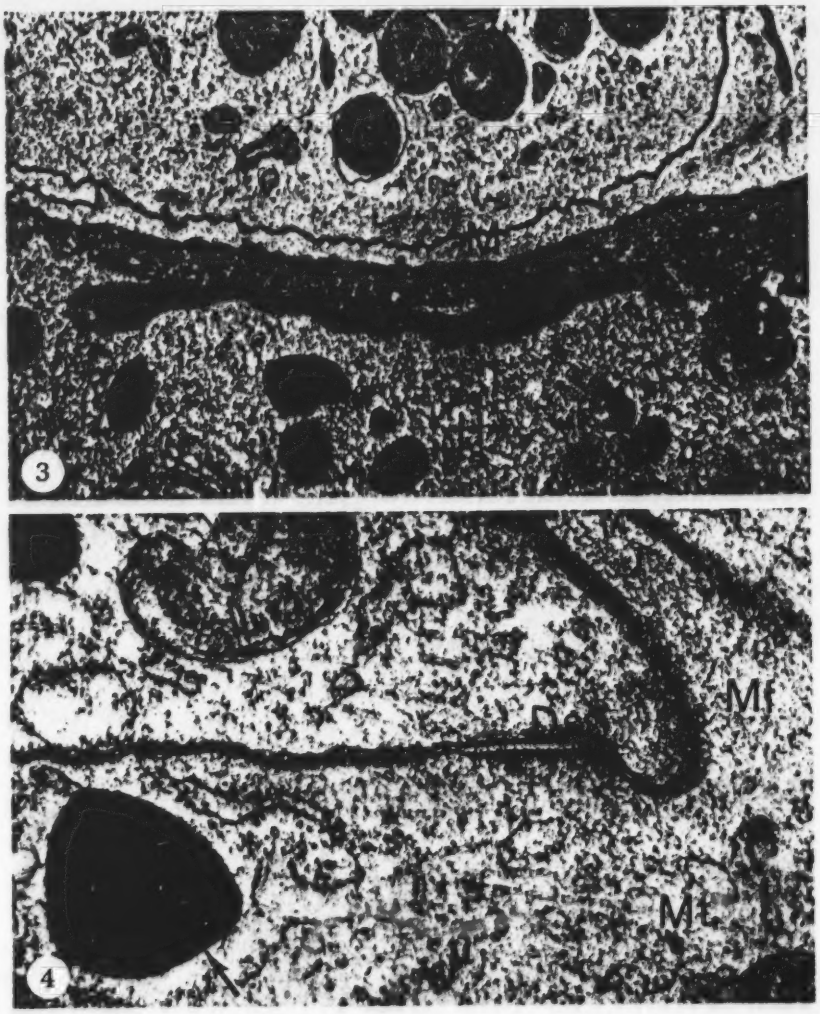


FIG. 3. Close accommodation of capsule hemocyte (H) to irregularities of the mesenteronic basal lamina (BL). A granule (G) characteristic of this type of hemocyte and *B. popilliae* bacilli (B) in mesenteronic tissue (M) are indicated. ×45,000.

FIG. 4. Cell membrane apposition between two closely cohering capsule hemocytes. Desmosome-like contact (Ds) and intermembrane gap are evident. Cell periphery microfilament network (Mf) and associated microtubules (Mt) are shown. Arrow indicates edge of granule sectioned at an angle favorable for visualization of limiting membrane. ×60,000.

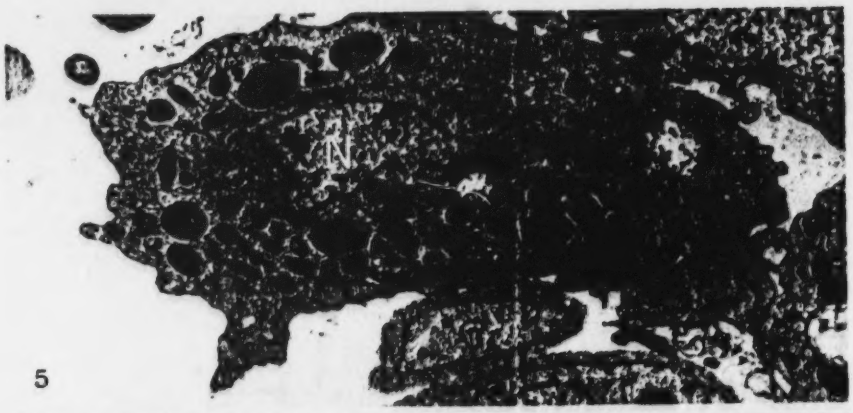
sity often suggestive of septa. The cytoplasmic faces of the apposed membranes at these contacts were thickened.

Hemocytes composing the capsule, with rare exceptions, were similar in ultrastructure. A conspicuous complement of angular, intensely staining cytoplasmic granules facilitated identification of these cells. When

FIG. 5. Hemocyte at the loosely organized, unattached capsule periphery. Note irregular surface contour and pseudopodia. N, Nucleus. ×9100.

FIG. 6. Tightly interdigitated capsule hemocyte. N, Nucleus; B, bacillus; and Gi, golgi. ×11,600.

FIG. 7. Granule-bearing hemocytes (H) surrounding clumps of bacilli (B) within mesenteronic tissue (M). ×7300.



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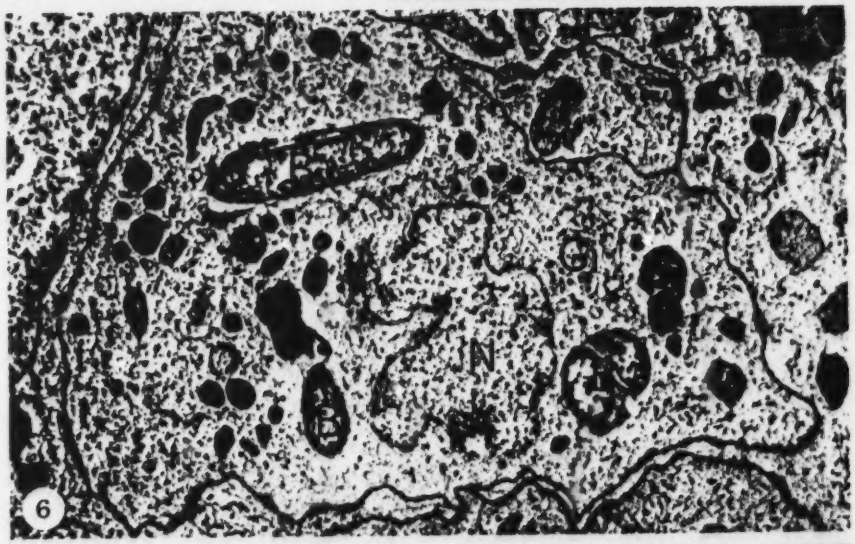

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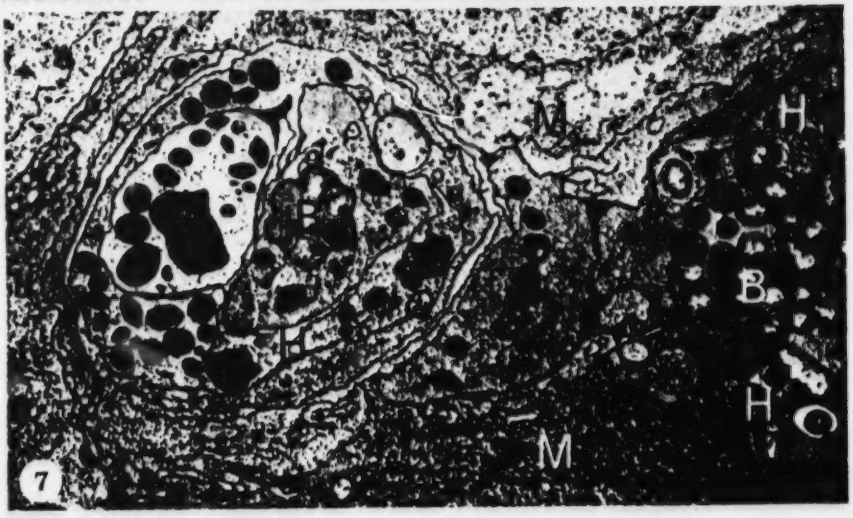

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FIGURES 5–7

planes of sectioning were favorable, the membranes delineating the edges of these inclusions were revealed (Fig. 4). Granule matrices were usually homogeneous and unstructured but infrequently peripheral microtubular substructure was observed. Nuclei were lobulate and heterochromatin was sparse. Mitochondria were few to moderate in number and Golgi and multivesicular bodies were frequent.

Cells at the unattached edges of the capsules appeared loosely incorporated. The outermost cells were amoeboid in appearance with irregular surfaces which bore numerous narrow pseudopodia (Fig. 5).

Hemocyte surface contours within the tightly interdigitated regions of the capsules were more regular (Fig. 6). Nucleoli and active Golgi appeared somewhat more frequently in these cells than in free hemocytes of the same type. These hemocytes as a rule were observed only within the hemocoel of controls but in challenged grubs they were encountered on several occasions on the lumen side of the basal lamina. In one lesion these cells were found surrounding clumps of bacilli within mesenteronic epithelium (Fig. 7).

Infected Cells

Infected mesenteronic cells and hemocytes contained heterogeneous arrays of cytoplasmic inclusions. Endocytosis of bacilli and their subsequent multiplication and/or degradation apparently result in the complexity.

Intracellular bacilli were nearly always sequestered in membrane-delimited vacuoles. Exceptions were in cells exhibiting advanced pathological changes where the continuity of the enclosing membrane could not be discerned with certainty. However, even in these cases bacilli free in the cytoplasm were only rarely observed.

Vacuoles were observed with bacilli in all phases of degradation. Often in the cytoplasm of such cells were dense vesiculate bodies and/or round osmiophilic bodies which in some cases appeared fused with vacuoles (Fig. 8). These inclusions appear to be secondary lysosomes.

Unique structures, resembling coils and parallel linear arrays of the outer lamina of the bacterial cell wall (Figs. 8–10), were usually observed in vacuoles of immature cells containing intact bacilli or in large bacterial aggregates in primary infection foci within regenerative nidi. Examination of such structures and transitional forms indicated that these configurations arise from sectioning of scroll-like entities at different angles. They were only rarely observed in hemocytes and their origin and significance remains unresolved.

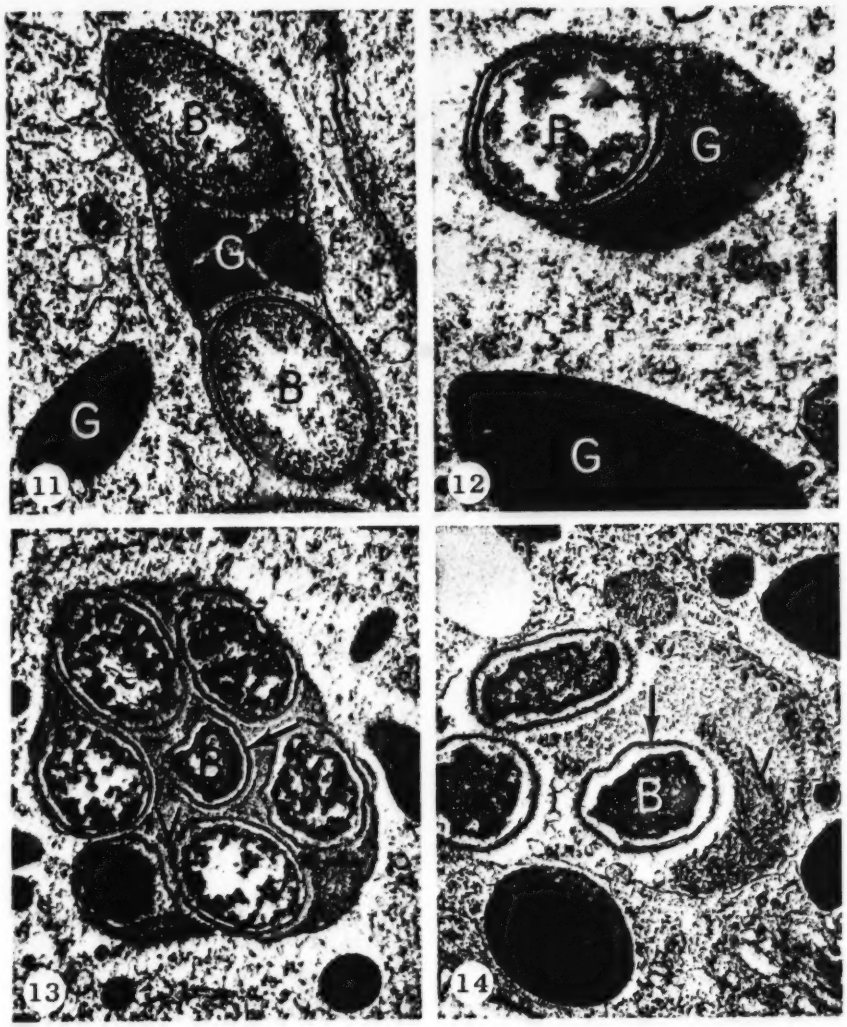
Large glycogen deposits flanking bacilli-bearing vacuoles were common in immature cells but infrequent in hemocytes and other types of cells. These infected immature cells usually possessed rough endoplasmic reticulum and their cytoplasms appeared diminished. The margins of these cells were characterized by the presence of a thick peripheral network of microfilaments below the plasma membrane (Fig. 8).

In hemocytes, granules identical to those in the cytoplasm were frequently present together with bacilli within vacuoles and endocytic invaginations of the plasma membrane (Figs. 11,12). Material closely resembling the matrix of intact granules was often observed together with deteriorating bacilli (Fig. 13). These findings are suggestive of a phenomenon similar to degranulation of vertebrate leukocytes. However, intravacuolar substances with other characteristics were also observed (Fig. 14).

Cells apparently overcame infections in some instances as evidenced by morphologically normal immature cells and hemocytes with residual bodies containing bacterial cell wall fragments. In other instances *B. popilliae* appeared to overcome cells (Fig. 15). Such cells often harbored numerous bacilli in flaccid vacuoles and possessed smaller nuclei and diminished cytoplasms containing fewer organelles. Occasionally cell lysis was observed.

FIG. 8. Noncolumnar mesenteric cell with glycogen (Gn) flanked vacuoles with bacteria (B) at various stages of degradation. Two structures, an osmiophilic body (OB) associated with a vacuole and a vesicular body (VB), possibly components of the lysosomal system are present in the cell. Arrows indicate scroll-like entities often present with large masses of bacteria. Microfilament network (Mf) at the cell periphery is also shown. ×19,000.

FIGS. 9,10. Transverse and longitudinal sections, respectively, of scroll-like structures found in association with large bacterial (B) masses. Note resemblance to part of the bacterial wall in Fig. 9. ×63,000; ×62,000.



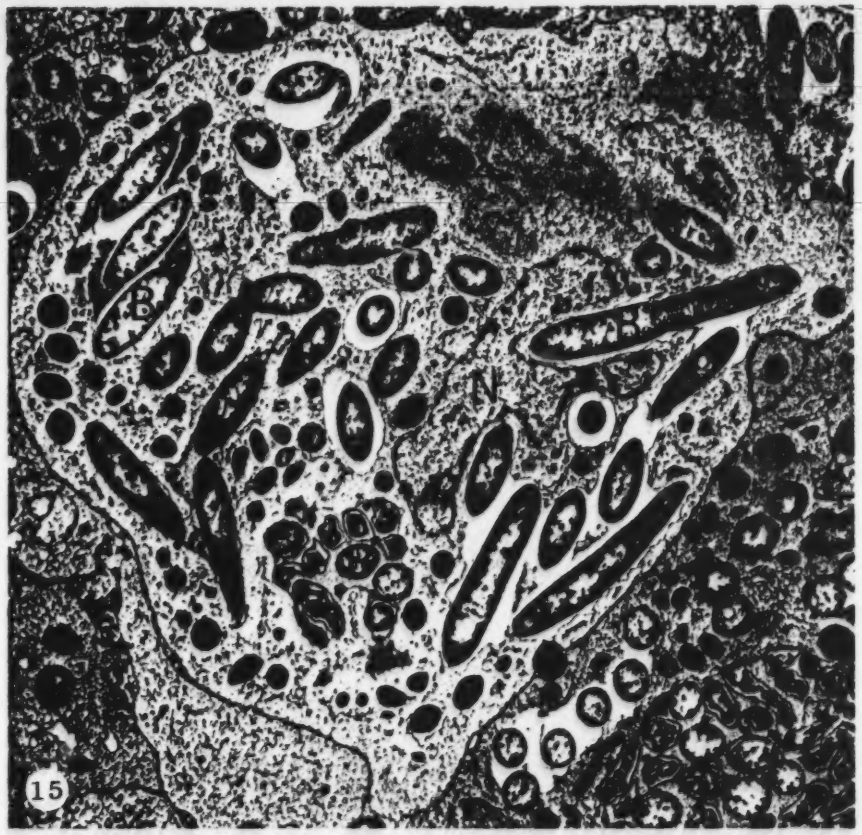
FIGS. 11,12. Hemocyte granules (G) in cytoplasm and in bacilli (B)-containing vacuoles. Bacillus in Fig. 12 has vacuolate appearance characteristic usually of degradative change within hemocytes. ×39,000, ×49,000.

FIG. 13. Intrahemocyte vacuole containing substance (V) with characteristics similar to those of intact granule matrix. Arrow indicates segments of bacterial (B) cell walls that appear doubled. ×24,000.

FIG. 14. Fibrillar intravacuolar substance (V) present with degraded bacilli (B) within granule-bearing hemocytes. The vacuolar content and agglomerated bacterial cytoplasm are atypical in hemocytes. Arrow indicates apparently duplicated region of the cell wall. ×24,000.

Bactericidal Activity of Cells

Bactericidal activity, manifested as alteration of bacterial structural integrity, was observed in both hemocytes and mesenteric cells. The degree of alteration appeared to correlate with the tightness of enclosure of bacilli within vacuoles. Generally, when bacilli were loosely enclosed, the vacuoles flaccid, and contact between vacuole membrane and microorganisms minimal or inapparent, the ultrastructure of the pathogens was either normal or only minimally altered.



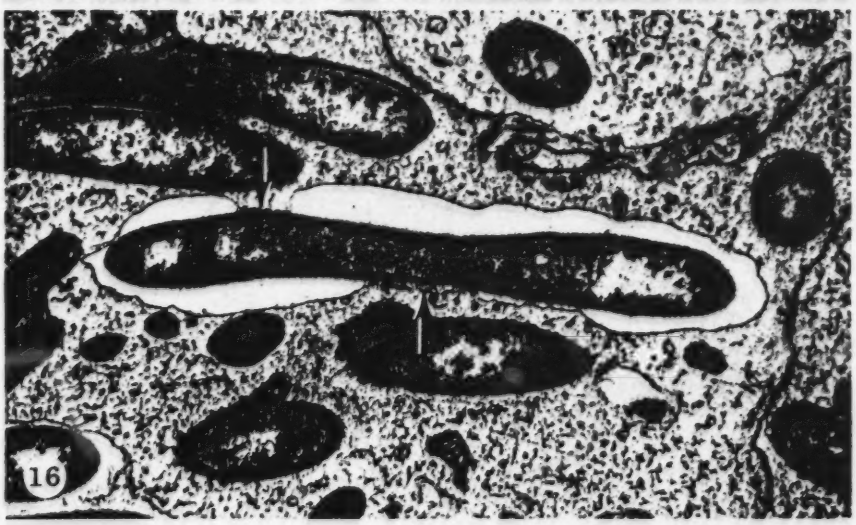


FIG. 15. Capsule hemocyte with attenuated nucleus (N) and diminished cytoplasm at an apparently advanced stage of infection by *B. popilliae* bacilli (B). ×8600.

FIG. 16. Arrows indicate regions of avid interaction between bacterial (B) fimbriae and vacuole membrane. ×18,000.

Strong affinity between bacterial fimbriae and vacuolar membranes (Fig. 16) was occasionally evident. Possibly as a consequence bacilli were usually more tautly contained within vacuoles. The frequency and degree of bacterial structural alterations observed in these instances were greater. However, not infrequently, both morphologically altered and normal bacilli were observed within a common vacuole.

Deteriorating bacteria usually exhibited varying degrees of one or more of the following alterations: (1) fimbriae loss, (2) retraction and/or alteration of cytoplasm, (3) diminishment of nuclear material, and (4) cell wall alteration such as loss of rigidity, fragmentation, dissolution, or apparent doubling of segments of the cell walls. The last cell wall alteration was one of the more notable changes. It appeared to result from an additional layer which ultrastructurally resembled the outer lamina of the normal cell walls (Figs. 13,14: arrows). Its formation appears to occur concomitantly with bacterial cytoplasm retraction. On occasion cell walls appeared to fragment but more commonly disintegration appeared to be gradual.

Differences in early degradative effects on bacteria were noted between mesenteronic cells and hemocytes. In immature mesenteronic cells agglomeration of bacterial cytoplasm into coarse electron-dense masses was usually observed (Fig. 8), while in hemocytes generally dissipation of nuclear material and central cytoplasm resulted in bacteria with vacuolate central regions and marginal electron-dense masses of remnant cytoplasm (Figs. 12,13). Agglomeration was also observed occasionally in hemocytes (Fig. 14). Later stages of bacterial degradation appeared similar in different cells. Bacterial structures gradually melded into a diminishing homogeneous electron-dense substance.

DISCUSSION

Regions of larval *A. majalis* mesenteron involved in *B. popilliae* invasion exhibit abnormal desquamation of columnar epithelium, induction of mitoses in regenerative nidi adjacent to and within focal lesions, increased numbers of noncolumnar cells, and formation of a hemocyte capsule subjacent to the infection (Splittstoesser et al., 1973, 1978). The increased cellularity at such loci adds to the complexity of the pathological alterations and makes histopathological interpretation difficult.

The present electron microscope study shows that the numerous noncolumnar cells in regions of infected mesenteronic epithelium consist of necrotic columnar epithelial and/or cells that are cytologically indistinguishable from undifferentiated and differentiating cells of normal regenerative nidi. Such observations are supportive of the conclusion that the altered histology results from epithelial infection and subsequent repair through epithelial regeneration. An analogous process occurs in vertebrate gut mucosa where desquamation and appearance of immature cells on the luminal surface of villi occur under various inflammatory conditions including acute bacterial infections (Takeuchi, 1971).

The basic roles of hemocyte phagocytic and encapsulation functions in insect defense mechanisms have been of much recent interest (Salt, 1970; Whitcomb et al., 1974; Maramorosch and Shope, 1975). The capsules in the present study formed during bacterial pathogen invasion of the mesenteron. Structurally the capsules are similar to those formed around metazoan parasites (Nappi, 1975) but most closely resemble those reported to develop in *Locusta migratoria* and *Melolontha melolontha* around parenterally injected foreign objects (Brehelin et al., 1975). Hemocyte layers proximal to mesenteronic lesions were more tightly interdigitated than the peripheral cells which were loosely incorporated. Organization of hemocytes into more or less well-defined zones as seen in *Ephestia kuehniella* capsules (Grimstone et al., 1967) was not observed in this study. However, intracellular desmosome-like contacts in

the more tightly organized areas of the capsules were similar to those reported in capsules of *E. kuehniella* and *L. migratoria* (Seureau, 1975).

Granule-bearing hemocytes formed the capsules in *A. majalis*. The characteristic membrane-delimited granules of these hemocytes were frequently observed within bacilli-enclosing endocytic invaginations of the plasma membrane and within vacuoles. Such observations are suggestive of degranulation as observed in other invertebrates (Cheng et al., 1975) and vertebrates (Stoessel, 1974) and may be indicative of an intracellular digestive function for these granules. To our knowledge this phenomenon has not been previously described in insects at the ultrastructural level.

The observed sequestration of intracellular *B. popilliae* within endocytic vacuoles in this study corresponds to findings with other epithelium-penetrating bacterial pathogens of insects (Davidson, 1973) and vertebrates (Savage, 1972). However, the insect pathogens, in contrast to those of vertebrates (Takeuchi, 1971), were at least partially susceptible to the bactericidal action of the host cells as evidenced by the alterations in their ultrastructure.

Differences in the early degradative effects on the bacilli within mesenteronic cells and hemocytes are suggestive of differences in bactericidal response in these tissues. This finding is not unexpected as one of the functions of phagocytic hemocytes is believed to be defensive. The large glycogen deposits flanking bacilli-containing vacuoles in immature cells of the mesenteron may be evidence of differences in metabolism during bactericidal action or, alternatively, glycogen neosynthesis from degraded bacterial components as observed in oyster hemocytes (Cheng and Cali, 1973; Cheng, 1975).

Neoplasia, inflammatory changes, and wound repair are often difficult to differentiate in invertebrates (Taylor, 1969). Mesenteronic changes and hemocoelic cellular aggregates somewhat similar to those observed in this study have been interpreted to arise from neoplastic change in regenerative nidi cells and subsequent metastasis into the hemocoel during protozoan invasion (Henry, 1967, 1969). However, there is convincing evidence for the inflammatory origin of hemocoelic cellular aggregates in other studies of similar pathogens (Brooks, 1970, 1971). In the present study resolution of ultrastructural differences between mesenteronic cells and hemocytes and orientive examination of adjacent thick and thin plastic sections with light and electron microscopes, respectively, made possible more definitive identification of cells. Consequently, we were able to ascertain that, although discontinuity of basal lamina between lesion and capsule was observed, only hemocytes infiltrated the mesenteronic tissue and evidence of regenerative nidi-derived cell invasion of capsule or hemocoel was never encountered. On this basis we conclude that the alterations observed in involved regions of the mesenteron arise from epithelial infection, regeneration, and inflammatory response of certain hemocytes.

ACKNOWLEDGMENTS

We wish to thank Bernadine Aldwinckle for her excellent technical assistance during the course of this study and Thomas M. Scotti, M.D., for his helpful suggestions during the preparation of the manuscript.

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